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Development of an Endogenous Virus–Free Line of Chickens Susceptible to All Subgroups of Avian Leukosis Virus

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SUMMARY. Primary chicken embryo fibroblasts (CEF) from special specific pathogen-free chicken lines are used for detection of contamination of adult or embryonic tissues, meconium, or tissue culture fluids with avian leukosis viruses (ALV). The suitability and efficiency of such tests depend on the susceptibility of CEF to the various subgroups of exogenous as well as endogenous ALV. The ideal CEF for such tests should be not only susceptible to all retroviruses, but also free of endogenous viruses so that such tests are immune to any interference that may occur between the endogenous and the tested (exogenous) viruses. CEF and/or chickens free of endogenous viruses are also desirable for gene transfer studies using retroviral vectors, such as RNA interference (RNAi) experiments and transgenic work. The absence of ev genes in CEF or chickens can empower clean detection of successful RNAi construct delivery or gene transfer. CEF free of ev genes are also essential reagents routinely used in growing and detecting unknown retroviruses in varied viral assays. This report documents the development of a new line of chickens, 0.TVB*S1, that is free of endogenous viruses and susceptible to all subgroups of ALV identified in chickens.

RESUMEN. Desarrollo de una línea de aves libres de virus endógenos, susceptible a todos los subgrupos del virus de leucosis aviar.

Los cultivos primarios de fibroblastos de embrión de pollo provenientes de líneas de aves libres de patógenos específicos son utilizados para detectar contaminaciones con el virus de leucosis aviar en tejidos embrionarios, meconio o cultivos celulares. La factibilidad y eficacia de este tipo de pruebas depende de la susceptibilidad de los fibroblastos de embrión pollo a los subgrupos de virus exógenos, así como a los virus endógenos. Los fibroblastos de embrión de pollo ideales para este tipo de pruebas deben ser no solo susceptibles a todos los retrovirus, sino también libres de virus endógenos de manera que las pruebas sean inmunes a cualquier interferencia que pueda ocurrir entre los virus endógenos y los virus exógenos para los que se realiza la prueba. Fibroblastos de embrión pollo y/o aves libres de virus endógenos también son deseables para estudios de transferencia genética utilizando vectores retrovirales, tales como experimentos con ARN de interferencia o experimentos transgénicos. La ausencia de genes de virus endógenos en aves o en fibroblastos de embrión de pollo puede mejorar la detección de la transferencia exitosa de moléculas de ARN de interferencia o de genes. Los fibroblastos de embrión pollo libres de genes de virus endógenos son reactivos esenciales utilizados rutinariamente en varios ensayos para la detección de retrovirus desconocidos. Este trabajo documenta el desarrollo de una nueva línea de aves designada 0.TVB*S1, que es libre de virus endógenos y susceptible a todos los subgrupos de virus de leucosis aviar identificados en pollos.

Key words: chicken line, free of endogenous viruses, ALV susceptibility

Abbreviations: ADOL = Avian Disease and Oncology Laboratory; ALV = avian leukosis virus; ALV-A = subgroup A of ALV; ALV-B = subgroup B of ALV; ALV-C = subgroup C of ALV; ALV-D = subgroup D of ALV; ALV-E = subgroup E of ALV; ALV-J = subgroup J of ALV; CEF = chicken embryo fibroblasts; ELISA = enzyme-linked immunosorbent assay; ev = endogenous virus; FC = flow cytometric; PCR = polymerase chain reaction; TVA = tumor virus A locus; TVB = tumor virus B locus; TVC = tumor virus C locus; TVJ = tumor virus J locus; RAV = Rous associated viruses; RBC = red blood cell

Avian leukosis virus (ALV) infections and expression are detrimental to the poultry industry (20,36,47,63). There are five well-defined exogenous subgroups (A, B, C, D, and J) of ALV, which are associated with malignant neoplasms in chickens (20,32,33,34,43,45,46,47,48,49,61,64). In addition, numerous subgroup E endogenous viruses are present in the genome of most chickens (3,4,6,8,9,13,15,25,28,37,38,40,50,59). The presence of endogenous virus in tissue culture cells can lead to contamination of vaccines (32,41,61) and to emergence of new recombinant viruses between exogenous and endogenous viruses (14,57).

Cellular resistance/susceptibility to exogenous and endogenous ALV is specifically determined by autosomal tumor virus (TV) loci, namely *TVA*, *TVB*, *TVC*, and *TVJ*, which carry alleles either encoding receptors mediating a specific subgroup of viral entry or blocking an entry. *TVA* encodes a membrane protein related to the family of low density lipoprotein receptors (12,30,62) and is mapped to chicken autosomal chromosome 28 (51). *TVB* is the most complex TV locus, and is related to the tumor necrosis factor (16). There are three *TVB* alleles, *TVB**S1, *TVB**S3, and *TVB**R. *TVB**S1 codes for susceptible receptors mediating infection by ALV subgroups B, D, and E and is dominant to both *TVB**S3 and *TVB**R; *TVB**S3 codes susceptible receptors for infection by subgroups B and D and is dominant to *TVB**R; *TVB**R encodes a truncated receptor, which permits neither B, D, nor E subgroups of ALV infection (1,65). *TVB* is cloned (2,16,42) and mapped to the chromosome 22 (53). *TVC* is shown to encode a protein most closely related to immunoglobin family. *TVC* is cloned and mapped to chicken chromosome 28, close to the *TVA* locus (29,31). *TVJ* is the latest identified locus and reported as the chicken Na^{+}/H^{+} exchanger type 1 (*chNHE1*) gene. *TVJ* encodes a 90 kDa cell surface protein and is mapped to chromosome 23 (17).

Avian leukosis viruses are retroviruses. Retroviruses have a tendency to integrate into germ line and are transmitted vertically thereafter to subsequent generations as endogenous retroviral

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sequences (19,20). There are reportedly more than 20 known subgroup E endogenous virus (ev) genes in the chicken, which encode for endogenous viruses or their components. Such genes are present in virtually all chicken genomes (50,52,58).

The nonessentiality of endogenous viruses for the chicken was first demonstrated by the identification of a fertile Leghorn rooster that lacked ev genes (5). This was supported by identification of similar chickens (18), and at least two lines have been developed free of ev genes (7,20,35). However, White Leghorn chickens that are free of ev genes are extremely rare (35), and with the exception of line 0, lines do not exist that have been characterized for susceptibility or resistance to specific subgroups of ALV.

Line 0 was the first developed chicken line characterized as free of subgroup E endogenous viruses, with resistance to subgroup E (C/E; *TVB**S3/*S3), and is maintained specific pathogen free. Line 0 has been available to the research community since the 1980s (7,9,27). Line 0 has been essential for detection of exogenous viruses in tissue samples from chickens and for eradicating exogenous ALV from breeder flocks following critical infections with ALV A in egg layers (27,39) and ALV J in broilers (33). Line 0 is also the source for a widely used immortalized cell line known as the UMNSAH-DF-1 cell line (44). This communication reports a recently developed endogenous virus–free line of chickens using line 0 and selected congenic chickens developed from line 0 (9). This new line of chickens is susceptible to exogenous as well as endogenous ALV (C/0; *TVB**S1/*S1), and is useful for detection of all endogenous and exogenous ALV.

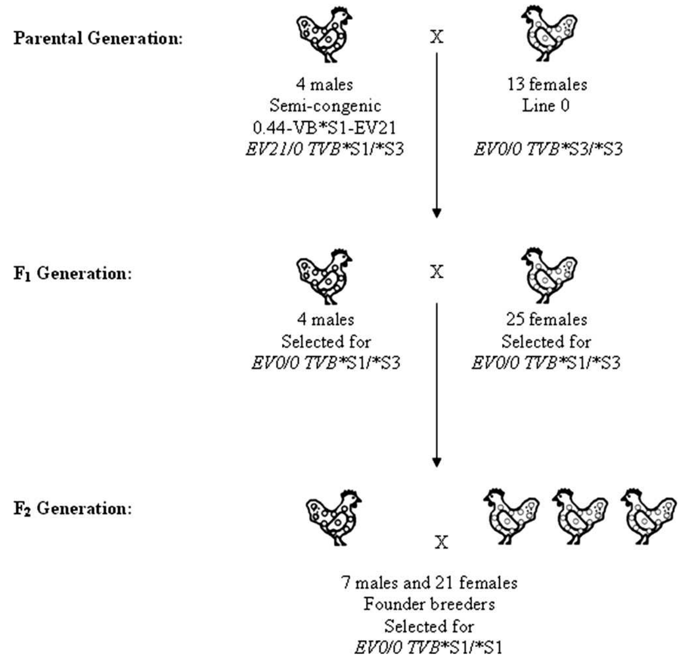


Fig. 1. The scheme of crossing and mating to produce ev-free F_1 birds with the desired *TVB* genotype (*TVB**S1/*S3) and lack of ev genes. The selected F_1 birds were mated *inter se* to produce an ev-free F_2 flock. Only F_2 birds with genotypes *EV0/0 TVB**S1/*S1 were selected as founder breeders for the new line 0.TVB*S1.

MATERIALS AND METHODS

Chicken lines. Line 0 was developed (as introduced above) and used in experiments at the ADOL by 1984 (22,27). In 1985 a male from the slow-feathering SPAFAS line 44 was mated to line 0 hens to obtain offspring to analyze ev genes (54). The ev gene *EV21* is tightly linked to the slow-feathering gene *K* on the Z sex chromosome (10). *TVB**S1 was also inherited from the line 44, and therefore *EV21* was expressed in line 0 chickens inheriting *TVB**S1. Males heterozygous for *EV21/0* and *TVB**S1/*S3 have been identified and mated to line 0 females to produce semi-congenic chickens with genotypes *EV21/0* and *TVB**S1/*S3 (7,9). The congenic nomenclature of these chickens was 0.44-VB*S1-EV21 indicating they were a line 0 chicken with *TVB**S1 and *EV21* genes introduced from the line 44 (9). To develop a semi-congenic line 0 of chickens homozygous for *TVB**S1, we inseminated line 0 hens with semen from the 0.44-VB*S1-EV21 males in 2004 and produced F_1 chickens in 2005. All the F_1 birds were subjected to R2 tests to detect the presence of EV21, and Pyrosequencing (Biotage AB, Uppsala, Sweden) to define *TVB* genotypes as described below. The F_1 chickens free of ev genes and heterozygous *TVB**S1/*S3 were selected and mated *inter se* to produce the F_2 chickens. Founder breeders were selected from the F_2 chickens (Fig. 1).

R2 assay. A hemagglutination test using R2 antiserum was done to examine the dual presence of cellular receptors capable of mediating ALV-E infection and expression of the *EV21* envelope gene in the red blood cells (RBCs) of all F_1 chickens (11). The F_1 chickens that tested negative by the R2 assay lacked endogenous viral expression (*EV*0/0 *TVB**S1/*S3), or lacked the cellular receptors susceptible to ALV-E (*EV*21/0 *TVB**S3/*S3), or both (*EV*0/0 *TVB**S3/*S3); those testing positive all possessed EV21 (*EV21/0*) and had the *TVB**S1/*S3 genotype.

Pyrosequencing analysis. *TVB* genotypes of all F_1 and F_2 birds from this experiment were determined by Pyrosequencing analysis following a previously reported procedure (65). Briefly, a short polymerase chain reaction (PCR) product is amplified from each of the DNA samples of the experimental birds with a reverse primer biotinylated at the 5′ end. The PCR products are then subjected to binding, shaking, annealing, washing, and denaturing processes. Finally, the PCR products are analyzed on a PSQ^{TM} 96MA Pyrosequencing system (Biotage AB) for the *TVB* genotypes. Among the F_2 experimental birds of this study, only three of the six commonly observed *TVB* genotypes are expected, i.e. *TVB**S1/*S1, *TVB**S1/*S3, and *TVB**S3/*S3. F_2 chickens with the *TVB**S1/*S1 genotype that were free of EV21 were selected for use as founder breeders in line 0.TVB*S1.

R2 FC assay. An R2 flow cytometric (FC) assay was performed to confirm the presence or absence of the cellular receptor on erythrocytes mediating ALV-B, ALV-D, and ALV-E entry following procedures described by Bacon *et al.* (11). This test incubates erythrocytes from chickens lacking ALV-E with plasma from chickens containing ALV-E followed by mixing the cells with R2 antibody and conducting FC analysis. RBCs of susceptible chickens (*TVB**S1/*S1 or *TVB**S1/*S3) binds ALV-E envelope glycoprotein 85 (gp85 from the plasma of chickens containing ALV-E). After incubation of this mix with the chicken R2 antibody, the gp85 is detectable indirectly with a fluorescein-tagged antibody to chicken immunoglobulin.

Southern blot analysis. Genomic DNA samples from each of the F_2 birds selected by Pyrosequencing analysis were digested with *Hin*dIII restriction endonuclease and separated by electrophoresis. Southern blot hybridizations were conducted as described previously (10) using a 279 bp fragment of the U3 region of the RAV-0 long terminal repeat (LTR) (20,26). DNA representing the RAV-0 U3 region was isolated from the U3N plasmid by *Bam*HI digestion. The U3 fragment was purified on agarose gel and labeled with ^{32}P using a random primer labeling kit (Stratagene, La Jolla, CA). This probe recognizes the LTR region of all ALVE endogenous viruses. Control line $15I_5$, EV21, and 7_1 DNA were from one chicken per line (9).

Preparation of chicken embryo fibroblasts (CEF). Primary CEF cultures were prepared from three 11-day-old embryos per selected founder breeder hen (9,21). Each CEF pool was prepared with three 11-day-old embryos of a breeder hen. Multiple secondary pools from each of the primary cultures per hen were then prepared for each of the 21 breeder hens selected to establish the new line 0.TVB*S1 and frozen in liquid nitrogen for subsequent ALV susceptibility analyses.

Analysis of ALV susceptibility. All hens but one were tested with two secondary CEF pools for each subgroup of the ALV, i.e., RAV-0, RAV-1, RAV-2, RAV-49, RAV-50, RAV-60, and Hcl. For hen number 16 only one pool was tested for each of the viruses. The viruses and the viral titers used in this test are given in Table 1. The viral titers ranged from $10^{2.2}$ for RAV-49 up to $10^{3.33}$ for the higher dosage of RAV-0. Two secondary CEF pools were prepared from each of the primary cultures of the selected founder breeder hens and were infected in duplicates with each of the six subgroups of ALV (A–E, and J) for all the six subgroups, separately. Rous associated viruses (RAV) used were RAV-1 (subgroup A), RAV-2 (subgroup B), RAV-49 (subgroup C), RAV-50 (subgroup D), RAV-0 and RAV-60 (subgroup E), and Hcl (subgroup J), respectively, to assess the susceptibility to ALV (8,33). The six subgroups of ALV and their titers used to infect the CEF in this study are given in Table 1. Eight days postinfection, supernatant fluids were tested for ALV group specific antigen (p27) by enzyme-linked immunosorbent assay (ELISA) as described by Smith *et al.* (56). OD_{630} values were obtained from 96-well microtiter plates. An ELISA OD value >0.2 indicates the CEF sample contains ALV. A positive ELISA test indicates the presence of p27, and that the CEF (and at least one of the parents of the tested embryo) is susceptible to the ALV used in the test. If the ELISA OD value is <0.2 the CEF lacks p27 and is resistant to that ALV (8). In addition to RAV-60, RAV-0 was also used as a secondary subgroup E virus to confirm the susceptibility of the CEF to ALV-E. The RAV-0 viruses with a titer of $10^{2.63}$ were used in an initial test of the CEFs from 10 breeder hens, which yielded average OD_{630} values smaller than 0.2. In the subsequent test for rest of the breeder hens, RAV-0 viruses with a higher titer, $10^{3.33}$, were used in the test to assess the susceptibility of the CEFs to ALV-E.

RESULTS

Production of F_1 chickens from a cross between line 0 females (*EV0/0 TVB*S3/*S3*) and 0.44-VB*S1-EV21 males (*EV21/0 TVB*S1/*S3*). Thirteen line 0 females were mated to four 0.44-VB*S1-EV21 semi-congenic males to produce F_1 chickens with four different genotypes: *EV0/0 TVB**S1/*S3, *EV0/0 TVB**S3/*S3, *EV21/0 TVB**S1/*S3, and *EV21/0 TVB**S3/*S3, with an expected ratio of 1:1:1:1. One hundred five F_1 birds were subjected to the R2 assay and Pyrosequencing analyses. All R2 positive F_1 birds (*EV21/0 TVB**S1/*S3) and R2 negative F_1 birds with genotypes of *EV21/0 TVB**S3/*S3 and *EV0/0 TVB**S3/*S3 were discarded. The F_1 birds that were R2 negative and *TVB**S1/*S3 were further examined for the presence of the *TVB* cellular receptors capable of mediating ALV-E infection by the R2 FC assay. Twenty-nine F_1 birds with the proper genotypes were then selected to produce the F_2 (Fig. 1). The selected F_1 birds were all free of ev genes and were uniformly heterozygous for *TVB**S1 and *TVB**S3 alleles (*EV0/0 TVB**S1/*S3).

Selection of founder breeder hens and roosters of specific genotypes (*EV0/0 TVBS1/*S1) from the F_2 flock that are free of ev genes.** Three different *TVB* genotypes were expected in the F_2 flock produced by the selected F_1 birds, namely *EV0/0 TVB**S1/*S1, *EV0/0 TVB**S1/*S3, and *EV0/0 TVB**S3/*S3 with an expected proportion of 1:2:1, respectively. Since all the selected F_1 birds were free of ev genes, Pyrosequencing analysis was initially conducted to identify the F_2 birds with the desirable genotype. One hundred sixteen F_2 birds were tested and 28 (seven males and 21 females) out of the 116 F_2 birds were identified as *EV0/0 TVB**S1/*S1 and selected as the founder breeders of the new line 0.TVB*S1.

Validation of ev-free status of the selected founder breeders. The R2 test had shown that all the selected F_1 birds used to produce the F_2 were free of EV21. Subsequently, all the selected founder breeder males and 17 of the 21 females were subjected to a Southern blot analysis utilizing pRAV-0 cDNA that hybridizes to all genes in chicken endogenous viruses to confirm their absence status of ev genes. Fig. 2 graphically confirms that all the tested founder breeder birds as well as the line 0 controls were free of ev genes, whereas the control birds from lines $15I_5$, 0.44-VB*S1-EV21, and 7_1 were positive for ev genes.

Table 1. Avian leukosis viruses and viral titers used in infections of the CEF.

ALV	Final titer of viruses used to inoculate CEF
RAV-1 (subgoup A)	$10^{2.87}$
RAV-2 (subgoup B)	$10^{2.63}$
RAV-49 (subgoup C)	$10^{2.2}$
RAV-50 (subgoup D)	$10^{2.33}$
RAV-0 (subgoup E)[A]	$10^{2.63}$
RAV-0 (subgoup E)[B]	$10^{3.33}$
RAV-60 (subgoup E)	$10^{2.33}$
ADOL-Hcl (subgroup J)	$10^{2.33}$

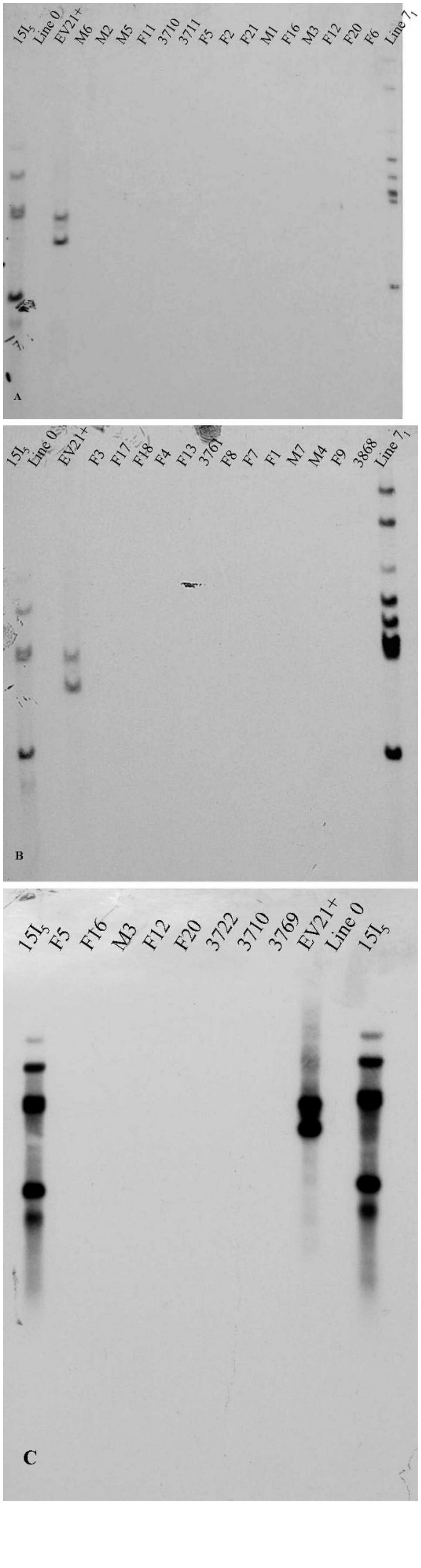
[A]Used to infect CEFs from the 0.TVB*S1 breeder hens 1–10.
[B]Used to infect CEFs from hens 11–21.

Evaluation of ALV susceptibility with CEF from the selected breeder hens. All the secondary CEF pools from the 21 founder breeder hens mated to seven breeder males tested positive (OD_{630} values >0.20) for each of the six subgroups of ALV with OD_{630} values ranging from 0.68 ± 0.013 to 1.22 ± 0.032 (mean $\pm$ standard error), except RAV-0. Two different titers of RAV-0 were used in the test. The CEF pools from the first 10 hens were tested with a lower titer of dosage ($10^{2.63}$), which resulted in negative ELISA results (OD_{630}: 0.00 ± 0.005 to 0.18 ± 0.103); the CEF pools from the other 11 breeder hens were tested with a higher titer of dosage ($10^{3.33}$), which then gave positive ELISA results (OD_{630}: 0.24 ± 0.058 to 0.58 ± 0.037). All the uninfected CEF pools tested negative. All positive control infected CEF cultures of line 15B1 tested positive for ALV. Line 0 infected CEF was also positive to all the known subgroups of ALV but negative for RAV-0 and RAV-60 as expected (Table 2). These tests confirm that all the selected founder breeders are susceptible to each of the six ALV subgroups.

DISCUSSION

Different subgroups of ALVs require different cellular receptors to initiate and complete the infection cycles. There are few reports on systematic characterization of the cellular receptors in commercial and special chicken lines, including those used for tissue culture–derived vaccine, or for the source of CEF to test ALV contaminations of breeders or vaccines. Some lines may be susceptible to certain subgroups of ALV but resistant to the others, or *vice versa*. Previously, chickens have been identified that were *TVB**S1/*S1 or *TVB**S1/*S3 and lacked expression of ALV-E; e.g., line 15B. However, these chickens possessed nonexpressed ev genes (20). The new line of chickens, 0.TVB*S1, was developed from line 0. It is homozygous *TVB**S1/*S1 and lacks ev genes. Furthermore, data from ELISA tests of the CEF pools prepared from embryos produced by the founder breeders confirms susceptibility to the six subgroups of ALV identified in chickens (Table 2).

It has been reported that among White Leghorn chickens, lines of chickens free of ev genes are extremely rare (35). Based on our search, we believe this new line of chickens, 0.TVB*S1, is the third line of chickens free of ev genes. The other two lines are the line 0 developed and maintained at the U.S. Department of Agriculture, Agricultural Research Service, Avian Disease and Oncology Laboratory and a Canadian line known as line WG (9,35). Line WG was shown by Southern blots to lack ev genes, but we are not



aware of its evaluation at the *TVB* locus. Line 0 is susceptible to all subgroups of ALV but resistant to ALV-E (C/E). The new 0.TVB*S1 line we have developed is the only one known to be free of ev genes, homozygous for the *TVB**S1 allele, and susceptible to all six subgroups of ALV (C/0). Embryos from line 0 and 0.TVB*S1 chickens are available to the research community upon request during the production season (December to July).

Several results appear somewhat variable and require further explanation. First, the Southern blots recorded in Fig. 2 show that the DNAs from all the founder breeders of line 0.TVB*S1 lacked bands when probed with pRAV2, whereas DNAs from chickens of control lines, $15I_5$, 7_1, and EV21, consistently had bands at the approximate expected positions based on previous tests (55). Variation in the relative density and spacing of the bands among Fig. 2A, B, and C was attributable to gel size and exposure time differences. The band numbers and relative spaces of the control lines $15I_5$, EV21, and 7_1 on Fig. 2A, B, and C were comparable.

The results for growth of subgroup E viruses were variable. RAV-60, which is a recombinant virus with subgroup E envelope (24), grew readily in the CEF from embryos of each of the 21 founder breeder hens. Based on OD values CEF from these hens were comparable with CEF from 15B1 embryos in growth of RAV-60. In contrast, RAV-0 did not grow when the inoculated titer was $10^{2.63}$ on CEF from embryos of hens 1–10 whereas it did grow on CEF from 15B1 CEF inoculated with an equivalent titer (Tables 1, 2). At a higher titer ($10^{3.33}$) RAV-0 did grow on CEF from embryos of hens 11–21 but not to the extent seen on 15B1 CEF (Table 2). These results indicate that line 15B1 CEF may be superior in growing normal endogenous viruses, like RAV-0, compared to CEF from the new 0.TVB*S1 line for reasons that remain to be explored. Line 15B1 is known to be superior for growth of ALV, particularly endogenous viruses, compared to other chickens or embryonic CEF (23). However, 15B1 contains EV1 and can produce recombinant viruses. Therefore, the new 0.TVB*S1 line is expected to provide the most suitable CEF for ALV isolation assuming the infection titer of viruses like RAV-0 is not lower than $10^{3.33}$. Further research is needed to define the lowest titer of RAV-0 which readily gives positive ELISA test results on the CEF of the line 0.TVB*S1 chickens.

This new line of specific pathogen free chickens should be of value as a constant source of CEF capable of growing ALV retroviruses or recombinants presently known to infect chickens in a variety of assays requiring tissue culture. The use of CEF from this new line should be particularly beneficial in tests of vaccine contaminations by retroviruses in contrast to CEF from chickens like line 0, which is unable to detect contamination by ALV-E or ALV-E recombinants

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Fig. 2. Images (A–C) of Southern blots analysis documenting the ev-free status for all 7 males and 17 of 21 females selected as founder breeders of the new line 0.TVB*S1. *Hin*dIII endonuclease digested DNA samples of the founder breeders were separated by electrophoresis. A pRAV-0 cDNA probe that hybridizes to all genes of chicken endogenous viruses was used to visualize the presence or absence of endogenous viral genes. As shown, the selected male (M) and female (F) breeders, and control birds from the line 0 were free of ev genes. The control birds from lines $15I_5$, 0.44-VB*S1-EV21, and 7_1 were positive for ev genes. Line $15I_5$ possesses *EV1*, *6*, *10*, and *15*; the semi-congenic chicken 0.44-VB*S1-EV21 possesses *EV21*; and the line 7_1 ev status is not defined, but it is known to possess ev genes (7). Lanes representing chickens 3710, 3711, 3761, 3868, 3722, and 3769 were F_2 *TVB**S1/*S3 nonbreeders that also lacked ev genes.

Table 2. Validation of susceptibility to ALVs for CEF from each of 21 0.TVB*S1 founder breeder hens and seven males by ELISA test (Mean ± SE of OD_{630} values).

Hen[A]	ALV[B]							Uninfected
	RAV-1	RAV-2	RAV-49	RAV-50	RAV-0[C]	RAV-60	ADOL-Hcl	
1	0.88 ± .030	0.81 ± .015	0.93 ± .020	0.93 ± .050	0.01 ± .005	0.86 ± .013	0.90 ± .021	0.00 ± .001
2	0.88 ± .027	0.87 ± .050	0.86 ± .038	0.89 ± .019	0.18 ± .103	0.84 ± .013	0.91 ± .025	0.00 ± .004
3	0.86 ± .029	0.85 ± .015	0.96 ± .055	0.83 ± .026	0.02 ± .005	0.85 ± .011	0.90 ± .018	0.01 ± .001
4	0.86 ± .028	0.78 ± .016	0.90 ± .017	0.83 ± .013	0.00 ± .005	0.89 ± .018	0.93 ± .009	0.00 ± .001
5	0.82 ± .020	0.78 ± .030	0.87 ± .014	0.84 ± .028	0.01 ± .005	0.94 ± .028	0.92 ± .038	0.01 ± .003
6	0.89 ± .017	0.84 ± .019	0.94 ± .021	0.86 ± .025	0.05 ± .025	0.88 ± .010	0.80 ± .077	0.00 ± .001
7	0.76 ± .014	0.73 ± .018	0.75 ± .015	0.80 ± .017	0.01 ± .010	0.95 ± .012	0.91 ± .007	0.02 ± .001
8	0.75 ± .016	0.73 ± .033	0.74 ± .042	0.92 ± .030	0.01 ± .002	0.91 ± .014	1.00 ± .024	0.02 ± .001
9	0.74 ± .013	0.68 ± .013	0.80 ± .023	0.99 ± .014	0.00 ± .005	0.71 ± .174	0.99 ± .021	0.02 ± .008
10	0.72 ± .014	0.74 ± .030	0.83 ± .010	0.98 ± .037	0.00 ± .005	0.86 ± .019	0.91 ± .045	0.01 ± .003
11	0.89 ± .082	0.88 ± .054	0.99 ± .100	1.04 ± .015	0.24 ± .058	0.95 ± .021	1.00 ± .029	0.02 ± .012
12	1.17 ± .025	1.11 ± .027	1.12 ± .003	1.1 ± .006	0.58 ± .037	1.04 ± .010	0.99 ± .006	0.10 ± .069
13	1.15 ± .024	1.12 ± .013	1.16 ± .029	1.22 ± .032	0.34 ± .042	1.01 ± .029	1.10 ± .016	0.01 ± .003
14	1.10 ± .017	1.18 ± .008	1.16 ± .007	1.13 ± .048	0.32 ± .060	1.03 ± .022	1.04 ± .013	0.03 ± .021
15	1.06 ± .005	1.14 ± .013	1.11 ± .007	1.10 ± .060	0.33 ± .034	0.99 ± .013	1.07 ± .044	0.01 ± .001
16	1.04 ± .004	1.09 ± .047	1.19 ± .006	1.01 ± .001	0.33 ± .053	1.10 ± .058	1.09 ± .016	0.01 ± .002
17	0.94 ± .024	0.99 ± .005	0.95 ± .014	0.92 ± .024	0.48 ± .023	0.91 ± .013	0.91 ± .023	0.00 ± .001
18	0.91 ± .036	0.99 ± .024	0.95 ± .061	0.94 ± .020	0.38 ± .047	0.87 ± .022	0.87 ± .020	0.00 ± .002
19	0.82 ± .019	0.94 ± .033	0.93 ± .019	0.89 ± .023	0.36 ± .146	0.88 ± .013	0.94 ± .039	0.00 ± .001
20	0.92 ± .032	1.00 ± .004	0.92 ± .018	0.88 ± .016	0.57 ± .053	0.91 ± .016	0.88 ± .018	0.00 ± .001
21	0.85 ± .007	0.94 ± .003	1.01 ± .026	0.86 ± .004	0.20 ± .020	0.92 ± .058	0.92 ± .046	0.00 ± .001
15B1	1.00 ± .046	0.97 ± .063	1.00 ± .025	1.01 ± .033	0.85 ± .025	1.03 ± .021	0.95 ± .035	0.02 ± .003
Line 0	0.90 ± .045	0.90 ± .054	0.95 ± .045	0.90 ± .018	0.00 ± .003	0.01 ± .006	0.93 ± .029	0.01 ± .002

[A]Egg fertilization was established by artificial insemination. A total of seven founder males were used in the insemination. Semen was used from each male to inseminate three founder females of the 0.TVB*S1 line. Pooled semen from males of line 15B1 and line 0 was used to inseminate hens from each of these lines. Primary CEF pools for each mating were prepared and frozen using three embryos per pool.

[B]From each primary CEF pool two plates of secondary CEF were subsequently infected with each of the prototype ALVs including RAV-1 (subgroup A), RAV-2 (subgroup B), RAV-49 (subgroup C), RAV-50 (subgroup D), RAV-0 and RAV-60 (subgroup E), and ADOL-Hcl (subgroup J). ALV growth was evaluated by detection of capsid p27 antigen in culture supernatants after 8 days using ELISA tests. OD_{630} values were obtained from 96-well microtiter plates. A value >0.2 indicates the CEFs are susceptible to the infected virus.

[C]Two different viral dosages were used. See Table 1 for details.

(32). Line 0.TVB*S1 also provides an optional source of embryos/chickens for gene transfer studies (RNA interference, transgenics, *etc.*) using retroviral vectors since the absence of ev genes evades potential interferences between exogenous and endogenous viruses of all ALV subgroups and empowers the detection of successful gene transfers.

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